# CHAPTER 3

# MATERIALS AND METHODS

Longan (*Dimocarpus longan* Lour.) fruit cv. Biew Kiew was harvested from Chiang Mai province, Thailand in the year 2007. The fruits were then separated into bunches with selected homogenous size and grading. The fruit bunches without defects and spoilage was used for the experiments.

The experiment design in this study was raid out in 2 x 2 x 5 Factorial in CRD with 4 replications. Treatments were including with no $SO_2$ treatment and $SO_2$ treated. Then, the treated fruits were stored at 2 ± 2 and 7 ± 2 degree Celsius. Finally, fruits were stored for 0, 2, 4, 6, and 8 weeks. The fruits were sampling immediately after $SO_2$ treatment at the rate 4.50 tons per 2.5 kg – $SO_2$, and then every month after stored under various storage temperatures as discussed above. The data corrections were followed;

### *3.1 Weight loss percentage*

The fresh weight of the fruit was determined for all treatments as an index of desiccation rate. Weight loss was calculated as following;

Weight loss = (Weight of fresh fruit – Weight of sample)/Weight of fresh fruit x 100

***3.2 Polyphenol oxidase activity and determination of pericarp pH***

Three fruits per treatment were thawed and peeled and 2 g of pericarp tissue was homogenized in 0.1 M phosphate buffer, pH 6.6 and 0.5 g of insoluble polyvinyl pyrollidone (PVP) (Merck) for 30 s with a polytron homogenizer (Kinematica GmbH, Kreins, Luzern, Switzerland; probe diameter, 20 mm). The homogenate was centrifuged for 10 min at 8000 x g in a Sorvall rotor SS-34 at 4$^{O}$C. The supernatant was collected and centrifuged repeatedly in 1.5 mL tubes at 20 000 x g for 10 min at 4$^{\circ}$C. The supernatant was collected into a fresh tube and 0.75 mL was used for the PPO assay in duplicate. The PPO assay was conducted by adding 0.12 mL 4-methyl catechol (Sigma, St. Louis, MO, USA) freshly dissolved (0.25 g) in 2 mL of ethanol and 10 mL of distilled water (final concentration, 23 mM). A control spectrophotometer was used to follow changes in absorbance at 410 nm over 2 min, and the linear progress of the reactions was recorded between 30 and 90 s. Protein content was determined according to Bradford (1976) with bovine serum protein as the standard. Results were calculated as in $\Delta$activity mg$^{-1}$protein x 1000. The change in the pH of the buffer was determined in duplicate. To determine the pH of pericarp and aril tissue, extraction was carried out as described above, but without PVP and with distilled water instead of phosphate buffer.



***3.3 Peel and aril color***

The pericarp (peel) and aril color of longan fruit were analyzed initially and after various storage duration. The color was measured on opposite sides of the fruit using (colourQuest XE, Hunter Associates Laboratory, Inc., New York, USA)

Minolta chromameter (model CR-200; Minolta, Ramsey, NJ) which provided CIE $L^*$, $C^*$, $H^*$, a*, and $b^*$ values.

***3.4 Preparation of longan pericarp for Scanning Electron Microscope (SEM), Light Microscope (LM), and Transmission Electron Microscope (TEM)***

The pericarps were cut in a dish of 0.1 M phosphate buffer pH 7.3. The pieces were transferred immediately after they were cut into a primary fixative solution. The longan pericarp pieces were fixed in a fixative solution as described by Bozzola and Russell (1999) with slight modification for anatomical study. The pericarp specimens were fixed with a primary fixative containing 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at 4°C for 2 hours. After that the tissue was usually washed in the same buffer vehicle used in the glutaraldehyde fixation step. Washing is extremely important because it eliminates any free unreacted glutaraldehyde that remains within the tissue. If aldehydes remaining from the primary fixation are oxidized by osmium tetroxide they may generate a "peppery" spot background and interfere in the specimens. Next, the specimens were post-fixed in 1% osmium tetroxide in the same buffer for 2 hours. Then, the specimens were dehydrated stepwise by exposure to ethanol-buffer mixture (30, 50, 70, 80, 90, and 100%) allowing 15 minutes in each, and critical point drying with liquid $CO_2$. This is a critical drying technique, as it achieves a phase change from liquid to dry gas without the effects of surface tension and is, therefore, suitable for delicate biological specimens for removal of water from the specimens. For SEM, the dried specimen was mounted on specimen studs and sputter coated with gold. Coated samples were stored in desiccator until assessed. Finally, the specimens were viewed with a

scanning electron microscope (JEOL, JSM-5910LV, JEOL Ltd., Tokyo, Japan) at 15 kV.

### *3.5 Sulphite residual (mg $kg^{-1}$)*

A sample of 50 g from the whole fruit, aril, and peel was obtained from a minimum of 30 fruits, and stored overnight at -70$^{O}$C. Sample were then examined in duplicate for sulphite residual according to de Vries *et al*. (1986).

### *3.6 The Quantification of Phenolic compounds*

*Sample extraction:* According to Nuchanart *et al* (2005); Freez – dried of peel and aril tissue of longan fruits cv. "Biew Kiew" and "DAW" were accurately weighted , 1.00 g, into 15 mL plastic tube (three replications per sample). 5.0 mL of 70% Methanol was added and vigorously shaken for 30 s. The samples were then left at room temperature overnight, with occasional shaking to ensure effectively extraction. Then, the samples were centrifuged for 3 min, at 25 degree C and 2 000 g, and the supernatants were transferred into 10 mL volumetric flasks. The residues were then re-extracted with 4.0 mL of 70% methanol. All extracts were combined and evaporated to final volumn about 2 mL. Finally, all samples were filtered through a 13 mm, 0.45 µm PVDF membrane (Orange Scientific, Belgium) prior to HPLC analysis.



*HPLC – UV/Vis Detection Analyse*: The HPLC analyses were performed using an HP 1100 HPLC system with a thermostatically controlled column oven, a binary pump, and a diode-array detector (Hewlett – Packard, Palo Alto, CA.). A 125 x 4 mm i.d., 5 µm reversed phase column, LiChrospher RP-18 was connected to a 4 x 4 mm

LiChrospher RP-18 guard column (Macherey-Nagel, Germany). The compounds were eluted with a gradient system of 0.4% formic acid (solvent A): methanol (solvent B) at a flow rate of 1.0 mL $min^{-1}$. The temperature of the column was 25 degree C with the UV detection at 270 nm. The injection volumn was 10µL. The gradient system started from 0 min (100%A) to 2 min (95%A), 5 min (70%A), 8 min (66%A), 11 min (45%A), 14 min (45%A), 17 min (100%A), and maintained at this ratio until 20 min.

*Calibration Curves*: All standards (gallic acid, and ellagic acid) were disloved in methanol to produce six concentrations at 0.008, 0.03, 0.01, 0.06, 0.10, and 0.20 mg $mL^{-1}$. The detection limit of each standard compounds was determind at the concentration where S/N ratio was >3.

*Recovery*: Crude samples were siked with known amounts of reference standard solutions (each concentration $n$ = 2), gallic acid (at concentration 0.05 and 0.08 mg $mL^{-1}$), and ellagic acid (at concentration 0.02 and 0.04 mg $mL^{-1}$) and then extracted following the method was previously described.

*Calculation of the phenolic compounds concentration*: The concentration of each phenolic compounds; gallic acid, and ellagic acid was calculated as following;

Phenolic compound concentration (mg $mL^{-1}$) = $(R_x / R_s)$ x $(C_s / W_x)$ x D

Where $R_x$ and $R_s$ are peak hights from test sample and standard, respectively; $C_s$ is concentration standard, µg mL; $W_x$ is weight of tested sample, g mL-1, and D is dilution factor (if solution injected is diluted).

***3.7 Measurement of membrane permeability***

Membrane permeability expressed by relative leakage rate, was determined according to the method of Jiang and Chen (1995). Discs were removed with a cork porer (10 mm in diameter) from the equatorial region of 30 fruits. Thirty discs (about 2 g) were rinsed twice and then incubated in 25 mL of 0.3M mannitol in distilled water at 25 degree C, and then shaken for 30 min. Electrolyte leakage was determined with a conductivity meter (Model PP-20, Sartorius). Another batch discs were boiled for 15 min in 25 mL distilled water and then cooled to 25 degree C to assess total electrolytes. The relative leakage was expressed as a percentage of the total electrolyte.

***3.8 Statistical Analysis***

The statistical analysis was carried out using a statistical program Statistix version released 8.0 and Least significant different test at 95% was used to determine significant difference among the treatments.